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ULTRASTRUCTURAL EFFECTS OF AAL-TOXIN T_A FROM THE FUNGUS *ALTERNARIA ALTERNATA* ON BLACK NIGHTSHADE (*SOLANUM NIGRUM* L.) LEAF DISCS AND CORRELATION WITH BIOCHEMICAL MEASURES OF TOXICITY

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H. K. Abbas, R. N. Paul, R. T. Riley, T. Tanaka and W. T. Shier. Ultrastructural effects of AAL-toxin T_A from the fungus *Alternaria alternata* on black nightshade (*Solanum nigrum* L.) leaf discs and correlation with biochemical measures of toxicity. *Toxicon* **36,** 1821–1832, 1998.—Ultrastructural effects of AAL-toxin T_A from *Alternaria alternata* on black nightshade (*Solanum nigrum* L.) leaf discs and correlation with biochemical measures of toxicity. In black nightshade (*Solanum nigrum* L.) leaf discs floating in solutions of AAL-toxin T_A (0.01–200 μM) under continuous light at 25°C, electrolyte leakage, chlorophyll loss, autolysis, and photobleaching were observed within 24 h. Electrolyte leakage, measured by the conductivity increase in the culture medium, began after 12 h with 200 μM AAL-toxin T_A, but was observed after 24 h with 0.01 to 50 μM AAL-toxin T_A, when it ranged from 25% to 63% of total releasable electrolytes, respectively. After 48 h incubation, leakage ranged from 39% to 79% of total for 0.01 to 200 μM AAL-toxin T_A, respectively, while chlorophyll loss ranged from 5% to 32% of total, respectively. Ultrastructural examination of black nightshade leaf discs floating in 10 μM AAL-toxin T_A under continuous light at 25°C revealed cytological damage beginning at 30 h, consistent with the time electrolyte leakage and chlorophyll reduction were observed. After 30 h incubation chloroplast starch grains were enlarged in control leaf discs, but not in AAL-toxin T_A-treated discs, and the thylakoids of treated tissue contained structural abnormalities. After 36–48 h incubation with 10 μM AAL-toxin T_A, all tissues were destroyed with only cell walls, starch grains, and thylakoid fragments remaining. Toxicity was light-dependent, because leaf discs incubated with AAL-toxin T_A in darkness for up to 72 h showed little phytotoxic damage. Within 6 h of exposure to $\geq$0.5 μM toxin, phytosphingosine and sphinganine in black nightshade leaf discs increased markedly, and con-

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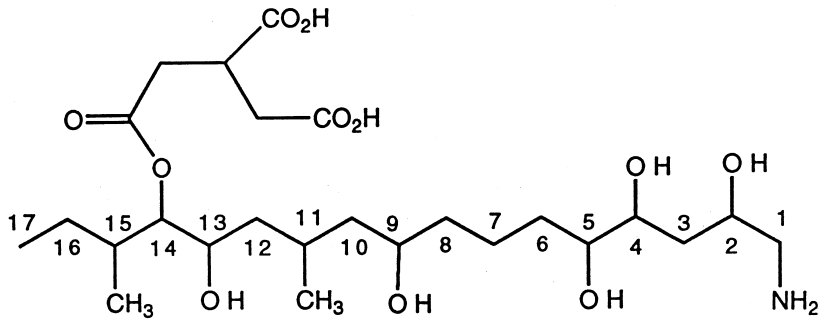
tinued to increase up to 24 h exposure. Thus, physiological and ultrastructural changes occurred in parallel with disruption of sphingolipid synthesis, consistent with the hypothesis that AAL-toxin T_A causes phytotoxicity by interrupting sphingolipid biosynthesis, thereby damaging cellular membranes.

INTRODUCTION

AAL-toxin T_A was first isolated from *Alternaria alternata* f. sp. *lycopersici* grown on liquid media (Bottini and Gilchrist, 1981; Bottini *et al.*, 1981). Subsequently, production of AAL-toxin T_A has been accomplished with other isolates of *A. alternata* including strain SWSL#1 grown on solid media (Abbas and Vesonder, 1993). AAL-toxin T_A has been well-documented to be phytotoxic to a variety of weed and crop species (Abbas *et al.*, 1993a, 1995b) even though it was initially thought to be a host-specific toxin for susceptible (*asc*/*asc*) tomatoes (Kohmoto *et al.*, 1982; Nishimura and Kohmoto, 1983; Mirocha *et al.*, 1992). Subsequent studies have shown that AAL-toxin T_A is highly phytotoxic to jimsonweed, black nightshade, prickly sida, duckweed and other higher plant species (Abbas *et al.*, 1993a,b, 1995a,b). AAL-toxins (Fig. 1) are chemically related to the fumonisins, including fumonisin B_1 (FB_1) which was initially isolated from *Fusarium moniliforme* MRC 826 grown on corn (Bezuidenhout *et al.*, 1988; Gelderblom *et al.*, 1988). They are both structural analogs of sphingosine consisting of long-chain alkylamines with propanetricarboxylic acid side chains (Bottini *et al.*, 1981; Bezuidenhout *et al.*, 1988). Studies in mammalian systems have demonstrated that FB_1 and AAL-toxin T_A act by inhibiting ceramide synthase, an enzyme in the sphingolipid biosynthetic pathway (Wang *et al.*, 1992; Yoo *et al.*, 1992; Merrill *et al.*, 1993b; Riley *et al.*, 1994a,b, 1996). Sphingolipids are important constituents of cell membranes (Merrill and Wang, 1986; Hannun and Bell, 1989; Merrill, 1991; Merrill *et al.*, 1993a; Shier, 1992). Inhibition of ceramide synthase in treated tissue was demonstrated by accumulation of the sphingolipid precursors, sphingosine and sphinganine (Wang *et al.*, 1992; Merrill *et al.*, 1993b; Riley *et al.*, 1994a,b). Previous studies have provided evidence that AAL-toxin T_A and FB_1 act by a similar mechanism of action in plant systems including duckweed (Abbas *et al.*, 1994), tomatoes and tobacco callus tissue (Abbas *et al.*, 1994), and corn seedlings (Riley *et al.*, 1996). The disruption of cell membranes can be measured in



AAL-toxin T_A

Fig. 1. Structure of AAL-toxin T_A.

cultured plants by cellular electrolyte leakage and by chlorophyll loss (Abbas *et al.*, 1994). AAL-toxin T_A and FB_1 were shown to cause elevation of the free sphingoid bases, sphinganine and phytosphingosine, prior to electrolyte leakage and chlorophyll loss. Studies of the ultrastructural effects of FB_1 on jimsonweed (Abbas *et al.*, 1992) and AAL-toxin T_A in susceptible tomatoes (Park *et al.*, 1981) have also provided evidence that these toxins cause membrane disruption. Park *et al.* (1981) reported that the first effects of AAL-toxin were on endoplasmic reticulum and mitochondrial membranes in the leaves of susceptible tomato plants, whereas they observed no effects in resistant tomato plants. However, the initial effect reported (Abbas *et al.*, 1992) for FB_1 treatment of cultured leaf discs of jimsonweed, a non-susceptible plant, was disruption of chloroplast envelope membranes.

Because commercially successful herbicides act by a very limited number of mechanisms, there is great interest in characterizing any novel phytotoxic mechanism to determine its potential to serve as a basis for the development of future herbicides. Consequently, we sought to examine in detail the mechanism by which inhibition of ceramide synthase leads to plant cell death as part of an ongoing research program to explore the potential of AAL-toxins or synthetic analogs with simplified structures (Abbas *et al.*, 1995c) as commercial herbicides. Additional study is necessary because it is not obvious how inhibition of sphingolipid synthesis can lead to cell death. In the present study we provide correlative evidence for the role of ceramide synthetase inhibition in the phytotoxic mechanism of a sphingosine analog mycotoxin by examining for the first time in the same experimental system the time sequence of toxin-induced accumulation of unutilized ceramide synthetase substrates and onset of ultrastructural effects.

MATERIALS AND METHODS

Plant material

Black nightshade (BNS) seeds were purchased from Thompson Seed, Fresno, CA, and planted in peat strips containing jiffy mix (Jiffy Products of America, Batavia, IL) and soil at a ratio of 1:1. The plants were watered as needed and the greenhouse temperature was maintained between 28 and 32°C with 40 to 60% relative humidity. The photoperiod was about 14 h at 1600 to 1800 $\mu E\ m^{-2}\ s^{-1}$ at midday. The primary and secondary leaves were used as experimental material when the plants were 2 to 3 weeks old.

AAL-toxin T_A

The AAL-toxin T_A (Fig. 1) used in this study was isolated and purified by the method of Abbas and Vesonder (1993) from the fungus *Alternaria alternata*, isolate SWSL#1 (NRRL 18822), grown on sterile rice medium. Purified AAL-toxin T_A was dissolved in sterile distilled water to yield a 200 μM solution. This stock solution was subjected to serial two-fold dilutions in distilled water to 0.01 μM (15 concentrations).

Electrolyte leakage

Fifty 2 mm diameter BNS true leaf discs were cut with a cork borer and washed in 1% sucrose containing 1 mM MES (2-[*N*-morpholino]ethanesulfonic acid) (pH 6.5) and then placed in a 6 cm-diameter polystyrene petri dish with 5 ml of wash medium with or without test compounds. Electrolyte leakage from the leaf discs was measured as described previously (Abbas *et al.*, 1992, 1994, 1995a). The dose response was carried out using 15 different concentrations (two-fold increases from 0.01 to 200 μM) of AAL-toxin T_A in triplicate and representative results from two experiments were combined. The discs were incubated with AAL-toxin T_A solutions at 25°C in continuous light for 48 h at 480 $\mu E\ m^{-2}\ s^{-1}$ in a growth chamber. At the beginning of the light period and periodically thereafter, conductivity of the bathing medium was monitored with a conductivity meter by sampling and returning bathing medium to each dish. Data is expressed as percentage increase in conductivity over controls. Control experiments were conducted with 0.01, 1.5 and 50 μM AAL-toxin T_A in either continuous darkness or continuous light.

Chlorophyll content

Chlorophyll was extracted after 48 h treatment with toxin or control, and assayed by the method of Hiscox and Israelstam (1979). Fifty leaf discs were soaked in darkness in 5 ml of DMSO at room temperature for 24 h, at which time chlorophyll extraction was complete. Sample tubes were centrifuged at $500 \times g$ for 10 min, and the absorbance of the supernatant was measured in a spectrophotometer at the wavelengths used for determining total chlorophyll with the equations of Arnon (1949).

Ultrastructural studies

Control samples were fixed in the same manner as treated tissue at each sampling time in order to monitor the effects of cutting the tissue into discs, floating them on a medium, and maintaining them in a growth chamber under continuous light (480 μE m^{-2} s^{-1}) at 25°C. Tissue samples from discs floating in 10 μM AAL-toxin T_A were harvested at 3, 6, 12, 18, 24, 30, 36 and 48 h after initial exposure to AAL-toxin T_A. The samples were fixed in 4% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, for 1 h. After a 30 min. wash (5 changes) in the buffer, the tissue was post-fixed for 1 h in 1% (w/v) osmium tetroxide in 0.05 M cacodylate buffer, pH 7.0. The samples were then washed for 30 min (6 changes) in distilled water. After serial dehydration in 25, 50, and 70% acetone (2 min each), the tissue was incubated in 70% acetone with 1% (w/v) *p*-phenylenediamine to assist in lipid retention (Ledingham and Simpson, 1972). After continuing dehydration in 70 and 90% acetone (2 min each), the samples were exposed to three 5 min washes in 100% acetone. The samples were embedded in Spurr's (Spurr, 1969) resin. Thin sections (80 nm) were cut with a diamond knife on a Reichert Ultracut E ultramicrotome (Cambridge Instruments, Buffalo, NY) and stained with 1% (w/v) uranyl acetate and Reynolds (1963) lead citrate. The sections were examined and photographed with a Zeiss EM10 CR transmission election microscope (Carl Zeiss, Thornwood, NY).

Determination of free sphingoid bases (phytosphingosine and sphinganine)

Leaf discs of BNS were used to determine the accumulation of free sphingoid bases over the time course during which cellular leakage (conductivity) and ultrastructural abnormalities occurred. BNS leaf discs (4 mm diameter) were cut with a cork borer from plants at the 6 to 8 leaf stage and washed in 1% sucrose, 1 ml MES (pH 6.5) and then placed in 6-cm diameter polystyrene petri dishes with 5 ml of wash medium with or without AAL-toxin T_A. Discs were incubated at 25°C under continuous light for up to 48 h at 500 μE m^{-2} s^{-1} in a growth chamber. Discs were examined visually for phytotoxic effects, and the conductivity of the bathing medium was monitored at each sample time. The experiment was conducted with 0, 0.1, 0.5, 1, 10, and 25 μM AAL-toxin T_A, and samples were taken at 6, 12, 24, and 48 h. At the appropriate times, leaf discs were removed, blotted with paper towels, frozen, freeze-dried, and stored at −80°C until they were analyzed for free sphingoid bases. Free sphingoid base determinations were performed in triplicate on groups of 50 discs and conductivity measurements made on the incubation media to confirm the time course of toxic responses. Extraction, purification, quantitation, and confirmation of sphingolipid metabolites were performed as described by Abbas *et al.* (1994).

RESULTS

Toxic responses of BNS leaf discs to AAL-toxin T_A

The toxic effect of AAL-toxin T_A in BNS leaf discs was readily demonstrated by electrolyte leakage into the culture medium and a decrease in chlorophyll content. Under continuous light at 500 μE m^{-2} s^{-1}, significantly increased electrolyte leakage into the culture medium was measured for BNS leaf discs within 6 h of treatment with all AAL-toxin T_A concentrations tested ($P < 0.005$, Student's unpaired *t*-test) (Fig. 2(A)). Electrolyte leakage increased rapidly with increasing AAL-toxin T_A concentration in the range 0–0.1 μM, above which there was little increase in electrolyte leakage with increasing toxin concentration. Dramatic increases in electrolyte leakage were observed after 24 h treatment with concentrations of AAL-toxin T_A higher than 0.1 μM.

A significant ($P < 0.005$, Student's unpaired *t*-test) decrease in chlorophyll content was also measured in BNS leaf discs under continuous light after 48 h for all 15 concentrations of AAL-toxins ranging from 0.01 to 200 μM. The percent decrease in chlorophyll content ranged for 5 to 32% from the lowest to the highest concentrations, respectively (Fig. 2(B)).

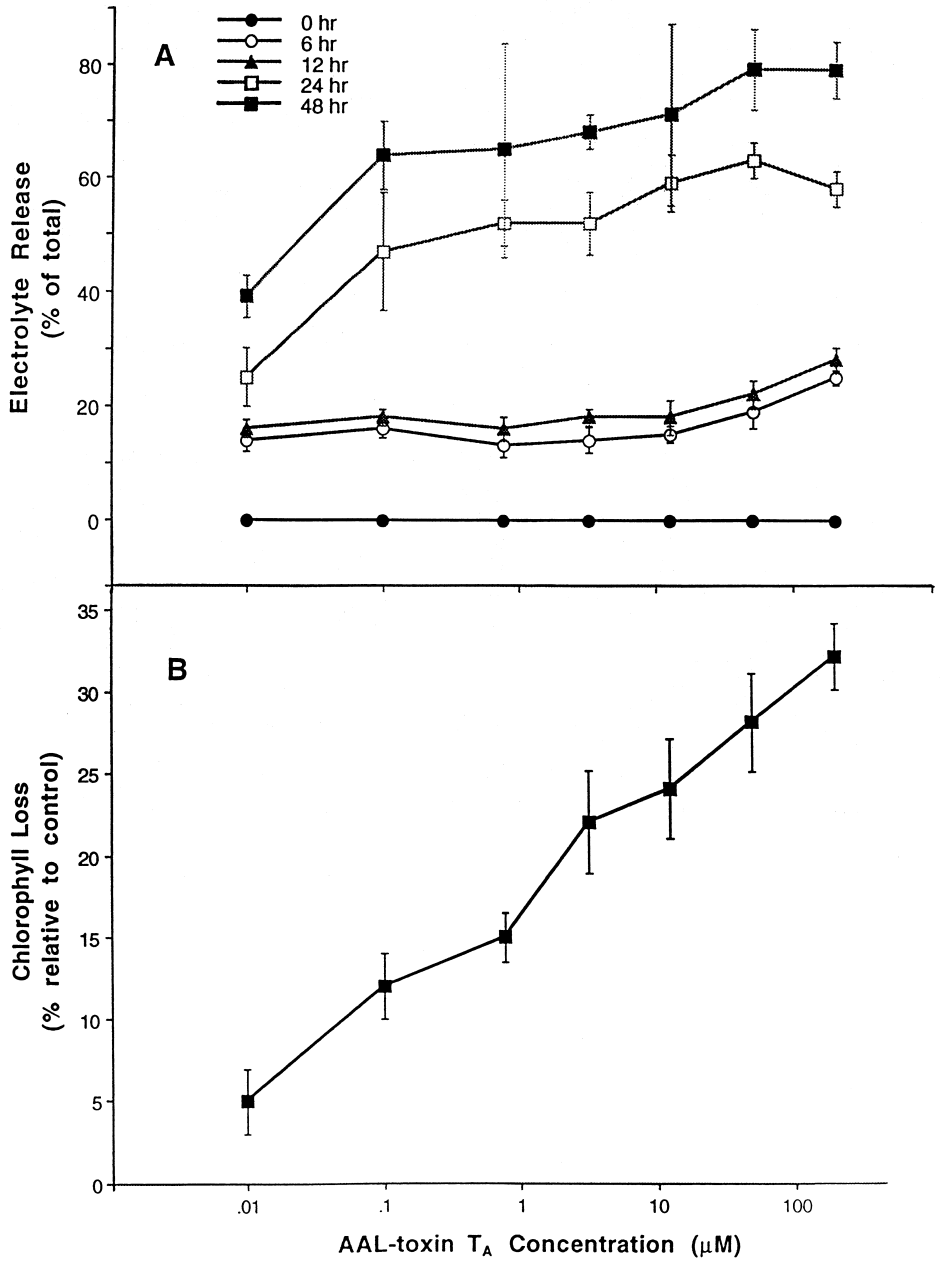


Fig. 2. (A) Effect of various concentrations (0.01 to 200 μM) of AAL-toxin T_A on electrolyte leakage from black nightshade (BNS) leaf discs during 48 h exposure to continuous light at 500 μE m^{-2} s^{-1} at 25°C as determined by the change in electrical conductivity of the bathing media with respect to untreated control discs. (B) Effect of the same concentrations of AAL-toxin T_A on chlorophyll content in BNS leaf discs, relative to controls. Values are the means of three replicates $\pm$ standard deviation at each time. All concentrations of toxin cause significantly greater effects than the toxin-free control ($P < 0.005$, Student's unpaired t-test).

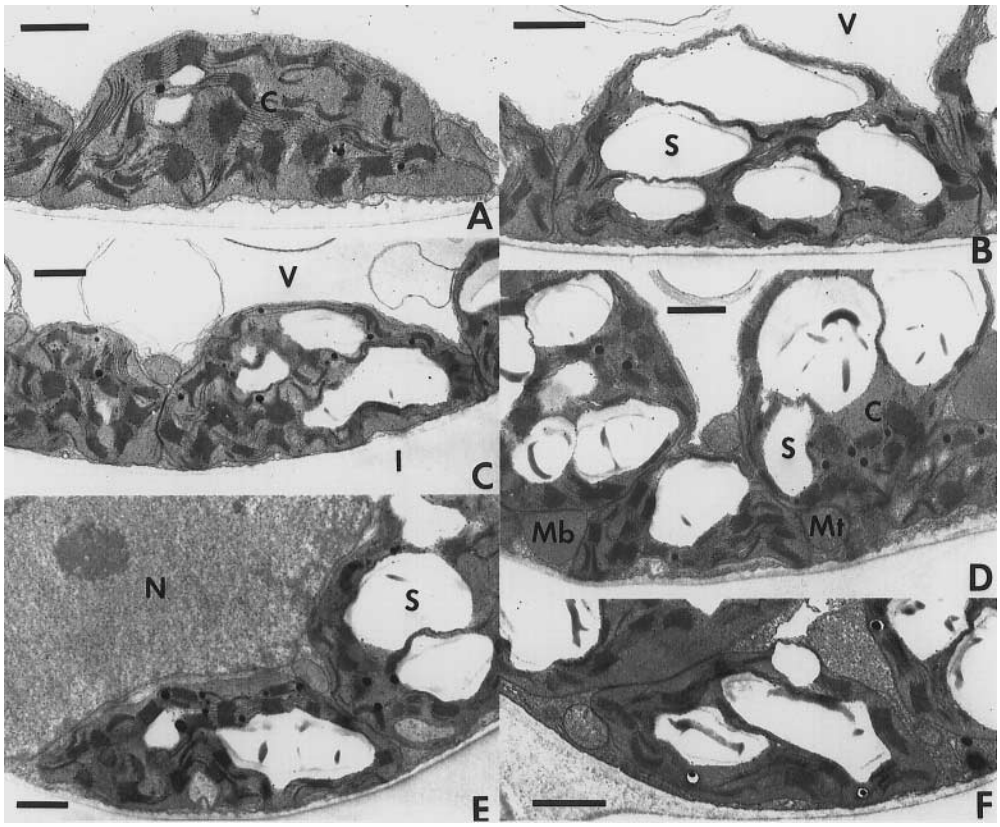


Fig. 3. Transmission electron micrographs of BNS palisade mesophyll chloroplasts sampled from leaf discs floating in distilled water for the indicated times (3–24 h). Little damage is evident in the treated tissue during the first 24 h of exposure to the toxin. (A) 3 h, treated; (B) 3 h, control; (C) 6 h, treated; (D) 12 h, treated; (E) 12 h, control; and (F) 24 h, treated. All bars = 1 μm. C = chloroplast; V = vacuole; S = starch grain; Mt = mitochondria;

Ultrastructural effects

Ultrastructural observation of BNS leaf discs treated with 10 μM AAL-toxin T_A (Figs 3 and 4) revealed a time course consistent with membrane damage measured as electrolyte release (Fig. 2). Very little difference was observed between treated and untreated control tissue at 3 h (Fig. 3(A) and (B)), 6 h (Fig. 3(C)), 12 h (Fig. 3(D)) and 24 h (Fig. 3(E) and (F)). At 12 h and later, starch grains were enlarged in both treated and control tissues (Fig. 3(E) and (F)). Beyond 30 h increasing cytological damage was observed in the treated tissue (Fig. 4(A–E)). In Fig. 4(A), the top cell appears normal except grana stacks in the chloroplasts have some abnormal conformations in the shape of circles or cups (Fig. 4(A), arrows). They are also more densely stained than in the corresponding controls. In Fig. 4(B), treated tissue at the same time of incubation reveals that the chloroplast envelopes have entirely disappeared, leaving the thylakoid system exposed to the cytoplasm. Total organelle disruption has not yet occurred, as evidenced by the intact mitochondrion (Mt). The cytoplasm itself remains densely stained which indicates the continued presence of ribosomes. Figure 4(C) shows typical damage due to the toxin after 36 h. The only recognizable structures other than the cell walls are starch grains and still-stacked grana. There are no recognizable organelles,

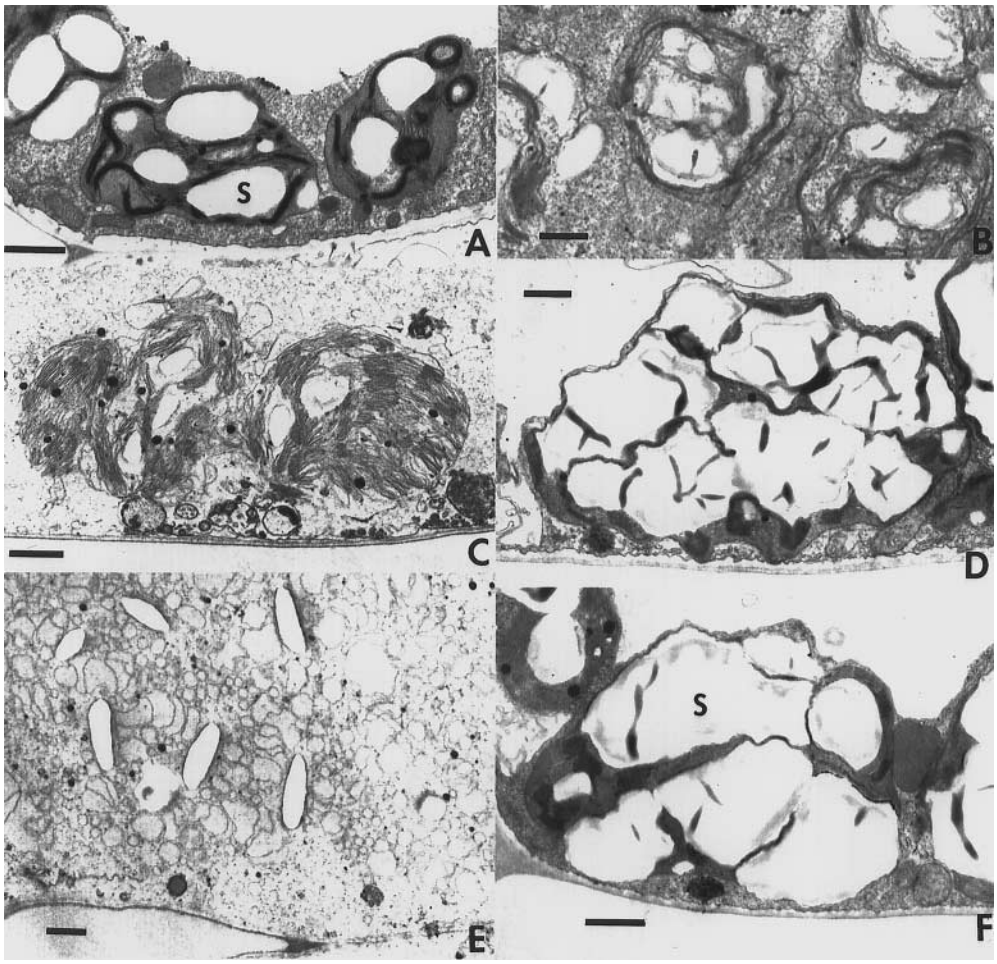


Fig. 4. Transmission electron micrographs of BNS palisade mesophyll chloroplasts sampled from leaf discs floating in distilled water (controls) or 10 μM of AAL-toxin T_A (treated) for the indicated times (30–48 h). (A) 30 h, treated; (B) 30 h, treated; (C) 36 h, treated; (D) 36 h, control; (E) 48 h, treated; and (F) 48 h, control. All bars = 1 μm. S = starch grain.

and ribosomes appear to be greatly reduced in number. At 36 h (Fig. 4(D)) and 48 h (Fig. 4(F)), control tissue, traumatized by cutting into discs, still maintains cytologic integrity with little change except the chloroplasts are greatly distorted by the continuing starch buildup. At 48 h of treatment with 10 μM AAL-toxin T_A (Fig. 4(E)), the tissues inside the cell walls are typically cytoplasmic remnants with no recognizable features except starch grains and membrane fragments. No organelles remain intact.

Light-dependence of toxic responses to AAL-toxin T_A in BNS leaf discs

AAL-toxin T_A caused visible photobleaching (change from green to light brown) of black nightshade (BNS) leaf discs incubated under continuous light within 24 h. The severity of bleaching increased with time and concentration of AAL-toxin T_A. The percentages of BNS leaf discs that were visibly bleached at 48 h were 10 ± 4.2, 25 ± 6.3, 85 ± 2.0, 90 ± 2.5 and $100 \pm 0.0\%$ when treated with 12.5, 25, 50, 100, and 200 μM

AAL-toxin T_A, respectively. Control BNS leaf discs remained green throughout the course of treatment. Minimal changes occurred in BNS leaf discs when incubated with AAL-toxin T_A under continuous darkness, such as softening and slight yellowing of the leaf discs at higher concentrations of the toxin (>1.5 μM).

AAL-toxin T_A-induced electrolyte loss from BNS leaf discs into the culture medium was similar after 48 h of continuous light and darkness (data not shown). However, chlorophyll loss at 48 h was significantly higher ($P < 0.005$, Student's unpaired *t*-test) for BNS at 1.5 and 50 μM AAL-toxin T_A in continuous light (Fig. 5). No significant dose–response was observed for AAL-toxin T_A-induced chlorophyll loss in the dark, but significant increases in chlorophyll loss with concentration increase were observed in the light ($P < 0.005$, Student's unpaired *t*-test).

Effects on sphingoid bases

In BNS leaf discs, levels of both free sphinganine and free phytosphingosine were significantly higher than control ($P < 0.005$, Student's unpaired *t*-test) after 6 h or more treatment with AAL-toxin T_A at 0.5 μM and higher concentrations (Fig. 6(A) and (B)). The accumulation of both free sphinganine and sphingosine increased with concentration of AAL-toxin T_A and time of exposure up to 24 h (Fig. 6(A) and (B)). At 48 h the level of sphinganine decreased and phytosphingosine leveled off. All AAL-toxin T_A concentrations from 0.5 μM and higher are significantly higher ($P < 0.005$, Student's unpaired *t*-test) than controls at 6 h and thereafter. At 24 and 48 h all AAL-toxin T_A concentrations are significant ($P < 0.005$, Student's unpaired *t*-test) relative to controls.

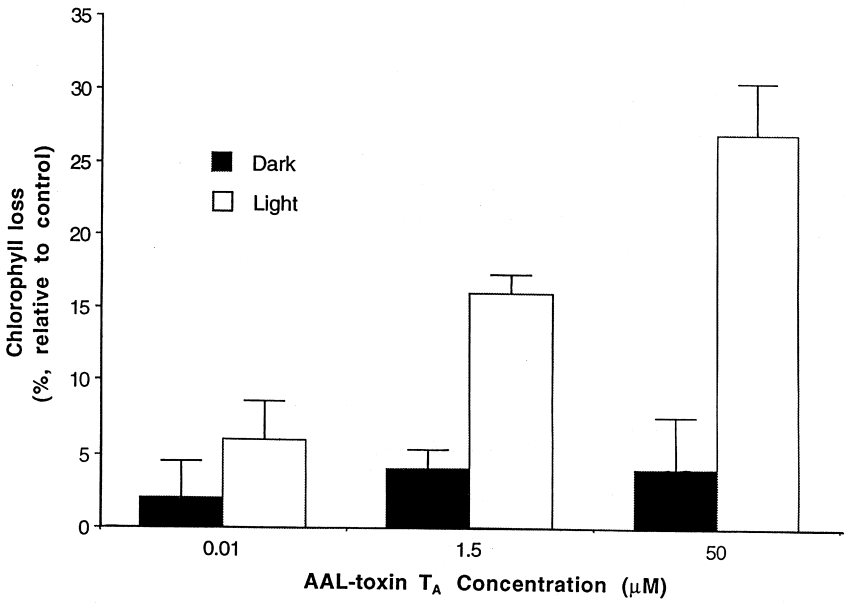


Fig. 5. The effect of light on the phytotoxicity of AAL-toxin T_A in black nightshade (BNS) leaf discs. Phytotoxicity of AAL-toxin T_A was measured by the reduction of chlorophyll content after 48 h incubation. There is no significant ($P > 0.05$, Student's *t*-test) chlorophyll loss in dark, but a significant loss ($P < 0.005$, Student's *t*-test) in light. Values are the means of three replicates $\pm$ standard deviation.

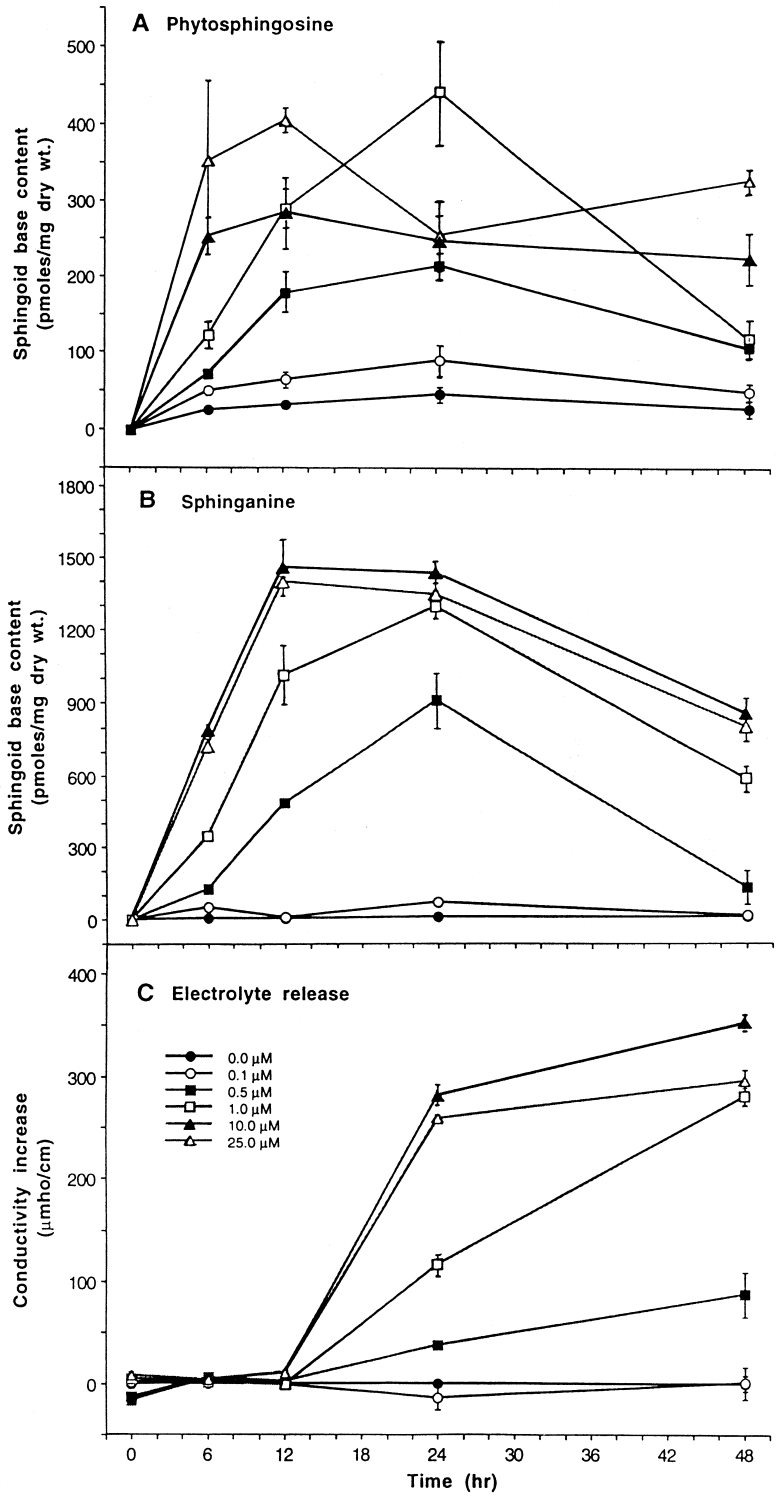


Fig. 6. The change in levels of free sphingoid bases in black nightshade (BNS) leaf discs treated with a range of AAL-toxin T_A concentrations. (A) Phytosphingosine levels in BNS tissues treated with the indicated concentrations of AAL-toxin T_A in the culture medium for various time periods. (B) Sphinganine levels in BNS tissues treated with AAL-toxin T_A for various time periods. (C) The concurrent change in conductivity of culture medium for the control and various AAL-toxin T_A concentrations. Values are the means $\pm$ standard deviation of three replicates at each time.

At 12 h only 10 and 25 μM AAL-toxin T_A are significant ($P < 0.005$, Student's unpaired *t*-test).

Increased culture medium conductivity caused by electrolyte leakage is a very early and sensitive measure of physiological phytotoxicity of many toxins, occurring before morphological symptoms can be detected visually or microscopically (Kenyon *et al.*, 1985; Duke and Kenyon, 1993). In BNS leaf discs, accumulation of free sphingoid bases occurred even before the first sign of toxin-induced phytotoxicity, increased culture medium conductivity with respect to controls (Fig. 6 (A), (B), and (C)). Significant increases in culture medium conductivity relative to controls ($P < 0.005$, Student's unpaired *t*-test) were observed after 12 h treatment for only the higher AAL-toxin T_A concentrations (10 and 25 μM) tested. However significant increases were observed at all AAL-toxin T_A concentrations tested at 24 and 48 h. Conductivity increased with increasing AAL-toxin T_A concentration and length of incubation.

DISCUSSION

The observed ultrastructural changes in cultured BNS leaf discs exposed to toxic concentrations of AAL-toxin T_A are similar to those reported (Abbas *et al.*, 1992) with FB_1, consistent with similar or identical mechanisms of action. However, the observed ultrastructural effects differ from those reported by Park *et al.* (1981) for incubation of intact leaves from a susceptible tomato strain with toxic material from *A. alternata*. The differences in results obtained may be due to (i) species-specific responses or different responses characteristic of susceptible strains of plants; (ii) the use of a crude toxin preparation by Park *et al.* (1981), which could have contained toxic levels of one or more other *A. alternata* toxins; or (iii) an effect of subtle differences in culture conditions.

During the time period used in the study of ultrastructural effects of AAL-toxin T_A on BNS leaf discs (Figs 3 and 4), the observed affects on membrane integrity appeared to follow a time course similar to electrolyte loss, specifically little effect at 6 and 12 h, major effects at 24 h and little additional effect in the subsequent 24 h. The time course of altered sphingolipid metabolism (Fig. 6 (A) and (B)) was distinctly different. Accumulation of sphinganine and phytosphingosine in AAL-toxin T_A-treated BNS leaf discs was the first change detectable after exposure to AAL-toxin T_A. Elevation of free sphingoid bases is consistent with inhibition of ceramide synthase, and it occurred during the lag period before major onset of ultrastructural and biochemical indicators of toxicity. This observation is consistent with inhibition of sphingolipid synthesis and turnover being the mechanism by which AAL-toxin T_A induces loss of membrane integrity, although the possibility of independent parallel induction mechanism(s) has not been eliminated. Previous studies (Hannun and Bell, 1989; Yoo *et al.*, 1992; Abbas *et al.*, 1992; Riley *et al.*, 1994a,b) in susceptible tomatoes (*asc*/*asc*) as well as in animals have demonstrated that AAL-toxin causes elevation of free sphingoid bases, which is consistent with inhibition of ceramide synthase. The observed light-dependence of AAL-toxin T_A-induced photobleaching, but not electrolyte release (Fig. 5), is consistent with chlorophyll loss being a more complex indicator of toxicity that is dependent on more than simply membrane integrity.

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